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ORIGINAL PAPER

Wolbachia endosymbiont is essential for egg hatching in a parthenogenetic arthropod

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Abstract *Wolbachia pipientis* can induce a range of sex ratio distortions including parthenogenesis. Recently *Wolbachia* has been detected in the diploid, parthenogenetic, collembolan species *Folsomia candida*. In this paper we address the effect of *Wolbachia* on reproduction in *F. candida*. *Wolbachia* infection was removed by antibiotic and heat treatment, and quantitative PCR techniques confirmed the success of the treatments. Complete loss of *Wolbachia*-infection led to the production of normal clutch sizes, but was associated with full egg hatching failure. Our results demonstrate that *F. candida* is strictly dependent on *Wolbachia* to produce viable offspring. This is one of the few cases of obligatory *Wolbachia* infection in arthropods. Our data suggest a unique mechanism underlying *Wolbachia*-dependence of egg development. One of our more salient results is that hatching success increased in consecutive egg clutches of antibiotic-treated individuals, probably due to restoration of bacterial densities over time. These observations suggest that reproduction in *F. candida* is a threshold effect requiring a critical *Wolbachia* density as is hypothesized by the bacterial dosage model. Quantitative PCR analysis showed that heat or antibiotic treated individuals with egg hatching failure had low average bacterial densities, but bacterial densities were not significantly different from those of treated individuals with successfully eclosing eggs. Additional experiments with partially cured *F. candida* are needed to prove the dosage model.

Keywords *Wolbachia* · Parthenogenesis · Bacterial dosage model · Egg hatching · Rifampicin treatment · Heat treatment

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Introduction

There is growing recognition of the evolutionary importance of symbiontic and parasitic micro-organisms in the manipulation of the reproductive mode of their hosts. Intracellular endosymbionts such as *Wolbachia*, *Cardinium* (Zchori-Fein et al. 1992), *Spiroplasma* (Hurst et al. 1999) and *Rickettsia* (Lawson et al. 2001; Werren et al. 1994) can induce a range of sex-ratio distortions, including cytoplasmic incompatibility, male-killing, feminization, and parthenogenesis. *Wolbachia*, a cytoplasmically inherited alpha-proteobacterium, infects many nematodes and arthropods such as Insecta, Collembola, Crustacea and Arachnida. *Wolbachia* is associated with the entire spectrum of reproductive phenotypes (Stouthamer et al. 1999; Werren 1997), but it is not understood if differences in the type of reproductive manipulation are due to different genetic backgrounds of the host or differences between *Wolbachia* strains (Stouthamer et al 1999).

The induction of parthenogenesis by *Wolbachia* infection is thought to be constrained to Hymenoptera and other haplo-diploid insects (Arakaki et al. 2001; Stouthamer et al. 1990; Weeks and Breeuwer 2001; Zchori-Fein et al. 2006). In these species treatment with antibiotics cures *Wolbachia* infection and leads to the production of males. *Wolbachia* is also found in parthenogenetic weevils of the genus *Otiorhynchus*. However, in this case the parthenogenetic weevils are polyploid, and there is little evidence that parthenogenesis is microbially induced (Normark 2003).

Recently, bacterial endosymbionts, including *Wolbachia*, have also been detected in the diploid parthenogenetic collembolan *Folsomia candida* (Willem) (Czarnetzki and Tebbe 2004b; Vandekerckhove et al. 1999). Collembola are soil arthropod species that are closely related to insects (e.g., Mallatt and Giribet 2006; Timmermans et al. 2008). Phylogenetic analysis using the 16S rRNA and *ftsZ* gene has placed the *Wolbachia* strain isolated from *F. candida* in a separate supergroup E that only includes collembolan hosts (Czarnetzki and Tebbe 2004a; Lo et al. 2002; Vandekerckhove et al. 1999). Additional screening for infected Collembola species has increased the number of strains in supergroup E, but has also refuted the exclusivity of *Wolbachia* supergroup E infection in Collembola by finding infections with supergroup A and B strains (Timmermans et al. 2004; J. Ellers, unpublished data).

Czarnetzki and Tebbe (2004a) found a relation between parthenogenesis and *Wolbachia* infection in a number of Collembola species; however, the effects of *Wolbachia* on *F. candida* are unknown and have only been speculated upon. Sex determination in the family of Isotomidae, to which *F. candida* belongs, is of the XX/XY or XX/X0 type (Dallai et al. 1999). *Wolbachia* causes feminization in the leafhopper *Zyginidia pullela*, which has the same sex-determining system (XX/XO; Negri et al. 2006). Feminization converts genetic males into reproductive females, often creating intersexes as a by-product (Bouchon et al. 1998; Negri et al. 2006; Werren 1997). However, the fact that virgin females can produce viable eggs makes it highly unlikely that feminization plays a role in *F. candida*.

A putative effect of *Wolbachia* in *F. candida* is the induction of parthenogenesis. However, the lack of *F. candida* free of *Wolbachia* prevents certainty about *Wolbachia*'s effect. Moreover, even if parthenogenesis is *Wolbachia*-induced, it is unclear if removal of *Wolbachia* causes *F. candida* to successfully shift to sexual reproduction. Sexual reproduction requires the production of males to ensure insemination, but in diploid–diploid species unfertilized eggs do not develop into males as they do in hymenopteran species. As a consequence, uninfected *F. candida* will remain unfertilized and oocytes are not expected to be viable. Therefore, removal of *Wolbachia* infection cannot provide conclusive evidence for its role in parthenogenetic reproduction in *F. candida*.

Alternatively, parthenogenesis may be unrelated to *Wolbachia*-infection, which then raises the question of whether *Wolbachia*-infection affects the host in other ways. All *F. candida* populations investigated so far are infected with *Wolbachia*. The only non-infected population reported to date is thought to be a different species based on molecular divergence data (Frati et al. 2004). The fixation of *Wolbachia* implies that these bacteria have become necessary endosymbionts for *F. candida* (Czarnetzki and Tebbe 2004a). Only a few examples of a transition of facultative to obligatory infection have been described for *Wolbachia* in arthropods, and all were in haplo-diploid species (e.g., Dedeine et al. 2001; Zchori-Fein et al. 2006). If *Wolbachia* is an obligatory endosymbiont of *F. candida* we expect removal of *Wolbachia* to severely decrease *F. candida* fitness by reducing survival or reproduction.

In this paper we want to examine the effect of *Wolbachia* on *F. candida* reproduction. We removed the *Wolbachia* endosymbionts by antibiotic and heat treatment and determined the success of the treatments using regular and quantitative PCR techniques. We assessed the effect of *Wolbachia* removal on reproduction by comparing clutch size and offspring viability of antibiotic-treated and untreated females. We will refer to females as uninfected if the presence of *Wolbachia* cannot be detected by regular PCR analysis using 16S rDNA primers.

Material and methods

Animals, treatment and quantitative PCR analysis

Folsomia candida females were taken from the laboratory culture ('Berlin' strain) of the department of Animal Ecology, VU University Amsterdam. This culture is used in many ecotoxicological and ecological studies.

In total about 120 animals were divided over six petridishes (Ø10 cm) with a moist plaster of Paris bottom to maintain humidity ($\sim$20 individuals per Petri dish). The animals were allowed to lay eggs on the Petri dishes for 72 h and were then removed. Beginning at 3–6 days after the eggs hatched, the Petri dishes were supplied with food for 28 days. Three Petri dishes were supplied with food consisting of a drop of yeast (*Saccharomyces cerevisiae*) mixed with water and $\sim$1% rifampicin (treated animals) and three Petri dishes were supplied with a drop of normal dry yeast as food (control animals). Food was replaced once a week with a freshly made mixture. After this initial period of the experiment, 20 animals were taken from each Petri dish and kept individually in small pots (Ø1.5 cm) and fed on dry yeast without rifampicin for the remainder of the experiment.

The efficiency of the rifampicin treatment was tested directly after the treatment using quantitative PCR. Total DNA was extracted from three animals from each Petri dish using the Wizard SV Genomic DNA purification system (Promega). DNA was dissolved in 50 µl nuclease free water. PCR reactions used 28S rDNA (*Folsomia candida*) and *ftsZ* (*Wolbachia*) primers and 3 µl DNA template. Primers used for amplification were: fols-28S-97f: GATGATGCGCCGCATTTATT and fols-28S-215r: CGCAGCAGTAGTTG GAAACCA (PCR efficiency 1.87), FtsZ-fols-142f2: ATTACAGCGGGAATGGGTGGTG and FtsZ-fols-241r2: TTGGCGCCCTATCCTTAACT (PCR efficiency 1.97). Each reaction was performed in duplicate using SYBR green mastermix (Applied Biosystems). Real-time amplification was performed on an Opticon thermocycler (MJ Research): denaturation (95°C for 15 min), two-step amplification and quantitation (92°C for 15 s, 60°C for 1 min, in total 40 cycles and one fluorescence measurement/cycle), melting curve program

(60–90°C, heating rate/s = 0.1°C, one fluorescence measurement/s). Differences between the Ct-values of the two duplicate measurements for each sample ranged between 0.3–0.9 and 0.08–0.3 Ct-value for *ftsZ* and 28S rDNA respectively. The comparative cycle threshold method (Livak and Schmittgen 2001) was used for relative quantification of the *Wolbachia* infections, using 28S rDNA of *F. candida* for normalization. By comparing the values obtained for the treated animals with those of the control groups we estimated the relative success of the antibiotic treatment.

Reproduction of rifampicin-treated *F. candida*

Animals were kept individually until they had laid three egg clutches or until they died. After oviposition of each clutch the adult animals were transferred to a new pot. For each individual, egg hatching success was scored per clutch as a binomial value, i.e., zero if no viable offspring were produced and one if at least a single offspring emerged. In addition, for each individual adult we counted the number of eggs of the first as well as the third clutch laid after the rifampicin treatment had ended. Clutches from the control animals were collected at the same time. Note that at 20°C *F. candida* reaches adulthood at about 17 days and individuals could have produced earlier clutches during the initial 28 days of the experiment, when they were not cultured individually. All animals that produced three clutches were analyzed for *Wolbachia* infection by PCR using *Wolbachia*-specific 16S rDNA primers: 16SAf –16SAr (Werren et al. 1995).

Exclude toxic effects of rifampicin on egg survival

To exclude the possibility that differences in hatching success between infected and non-infected *F. candida* were due to toxic effects of rifampicin on egg development, *Wolbachia*-infection was also removed from different *F. candida* adults using a heat treatment. More than 200, 2–6-day-old juvenile *F. candida* were kept at 30 ± 1°C for 30 days in a heating oven. Mortality was very high with only 17 animals surviving the full time interval of 30 days. After heat treatment, animals were allowed to develop individually in small pots under normal conditions (20°C 75% RH 12/12 LD) with dry yeast to feed on. Hatching success of the first clutch laid after transfer to individual pots was scored as a binomial value for each individual. After oviposition, the animals were frozen at −20°C and tested for *Wolbachia* infection using PCR with 16S rDNA primers (as described above).

Bacterial infection density

To test whether the density of the bacterial infection influenced egg hatching, all antibiotic-treated animals that survived until the end of the experiment were subjected to quantitative PCR analysis with the 28S rDNA and *ftsZ* primers described above, with minor adjustments: the reactions were performed in triplicate. Per individual, 3 µl template DNA (out a total of 100 µl) was amplified in a total volume of 20 µl reaction mix consisting of 10 µl SYBR green (Applied Biosystems), 0.5 µl of each Forward and Reverse primer (10 mmol) and 6 µl H_20.

Statistics

We analyzed the effect of rifampicin treatment on clutch size using a general linear mixed model Analysis of Variance (ANOVA) with treatment as fixed factor and replicate as

random factor. To analyze the effect of rifampicin on hatching success of the clutches, the percentage of clutches from which at least one egg hatched was calculated per replicate for the first, second and third clutch. The data was arcsine-transformed to obtain a normal distribution. We then performed a general linear model ANOVA with treatment as the main factor and clutch rank as covariate. A χ^2-test was applied to determine if egg hatching success differed between successive clutches of the rifampicin-treated and control females. To analyze whether egg hatching was associated with infection status of the rifampicin treated and heat-treated *Folsomia* females, we used Fisher's exact test as some cells on the two by two contingency tables contained fewer than five observations (Quinn and Keough 2002).

The software program Q-Gene (Simon 2003) was used to analyze the quantitative PCR data on bacterial density and to calculate Mean Normalized Expression values ("Procedure 2"). In several samples the standard error of the mean exceeded 20% of the mean normalized expression value (which is the default Q-Gene threshold), either because some individuals were marginally infected, or because one of the three replicates had a deviating value. Exclusion of deviating replicates did not affect our conclusions and therefore we present the analysis with all samples included. Animals were divided into three groups based on the 16S rDNA analysis: (1) infected with hatching eggs; (2) infected without hatching eggs, and (3) uninfected without hatching eggs. The resulting dataset was analyzed using a non-parametric (*K* independent samples) Kruskal–Wallis test. An additional non-parametric (two independent samples) Mann–Whitney test was performed to test whether the group of infected animals without hatching eggs (group 2) showed lower bacterial densities than the infected animals with hatching eggs (group 1).

Results

To remove *Wolbachia* infection from *F. candida*, animals were fed yeast mixed with rifampicin. The efficiency of this treatment was determined by means of quantitative PCR. *Wolbachia ftsZ* was greatly reduced in the treated animals compared to the controls, showing that rifampicin was effective. The normalized Ct values obtained for two of the treated groups were 7.9 (rifampicin 1) and 10.9 (rifampicin 2) cycles higher than the average of the untreated animals, which suggests that *Wolbachia* densities in the treated animals was $\sim$230 and $\sim$1,900 times lower. In addition, no *ftsZ* product was obtained after PCR on animals taken from the last rifampicin treated group (rifampicin 3) (SYBR green signal did not reach the threshold) while the values for 28S rDNA were in the expected range, suggesting almost complete removal of the endosymbionts.

Figure 1 shows the average clutch size of rifampicin-treated and control animals placed individually in small pots. Although there is some variation in number of eggs within and between replicates, the number of eggs laid by rifampicin-treated animals is not significantly different from the number of eggs laid by control animals (Table 1). Therefore, no evidence was found for a negative effect of the prior rifampicin treatment on egg production or oviposition.

In contrast, rifampicin treatment strongly affected the hatching success of eggs. The number of clutches from which eggs hatched was significantly lower in the treated group (ANCOVA on arcsine transformed hatching success with treatment as main factor: $df = 1.15$, $F = 61.8$, $P < 0.001$; and clutch rank as covariate: $df = 2.15$, $F = 4.0$, $P = 0.063$; Fig. 2). Hatching eggs were observed in 0–40% of the clutches of the treated group, compared to 90–100% of the clutches of the control group. The hatching success

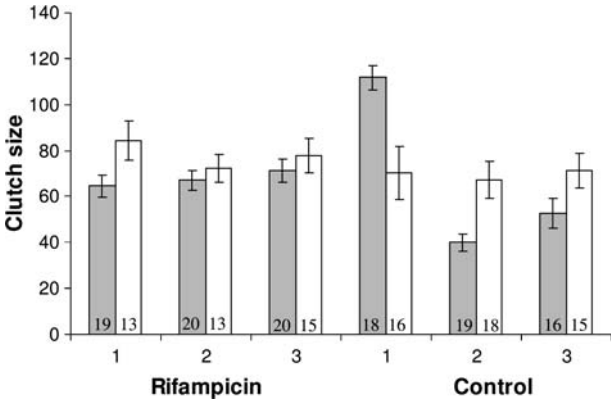

Fig. 1 Number of eggs (mean ± SE) laid in the first (*shaded bars*) and third (*white bars*) clutches of *Folsomia candida* in the rifampicin and control treatment. Although significant variation is found between replicas in first clutch size, the clutch size does not differ between treatments at any time. Numbers given in bars represent number of individuals included

Table 1 ANOVA of rifampicin vs. control treatment of *Folsomia candida* on first and third clutch size

	First clutch			Third clutch		
	df	*F*	*P*-value	*df*	*F*	*P*-value
Rifampicin treatment vs. control	1	0.00	0.983	1	5.50	0.074
Replica (treatment)	4	28.1	**<0.001**	4	0.28	0.893
Error	99			75		

Each treatment was performed in triplicate with 20 individuals each; sample sizes in the analysis of third clutch size are slightly reduced because of mortality occurring during the experimental period (36 days)

Significant *P*-values are shown in bold

was lowest for the group for which the *Wolbachia* removal was most successful (treatment 3). Unexpectedly, a comparison of the hatching success of the first clutch to hatching success of the second and third clutches showed that the hatching success of clutches of the treated animals increased over time ($\chi^2 = 17.8$, $P < 0.001$ and $\chi^2 = 41.6$, $P < 0.001$ respectively; Fig. 2). For the third clutch, hatching eggs were observed in 15–80% of the clutches. The gradual recovery of the hatching success was only observed in the rifampicin treated animals, not in the control group (clutches 1–2: $\chi^2 = 0.52$, $P = 0.471$ and clutches 1–3: $\chi^2 = 1.92$, $P = 0.166$ respectively; Fig. 2).

PCR analysis using 16S rDNA primers revealed that successful egg hatching was restricted to the infected animals (Table 2). All 17 individuals that remained uninfected until the end of the experiment failed to produce viable offspring. In contrast, of the 24 treated but infected individuals, 19 produced viable offspring. The distribution of successful egg hatching over infected and non-infected individuals deviated significantly from random (Fisher's Exact test, $P < 0.001$). We may therefore conclude that hatching success depends on *Wolbachia* infection status.

To exclude the possibility that differences in hatching success were due to potentially toxic effects of rifampicin on egg development, *Wolbachia* infection was also removed using heat treatment. The infection status of *F. candida* after the treatment was again

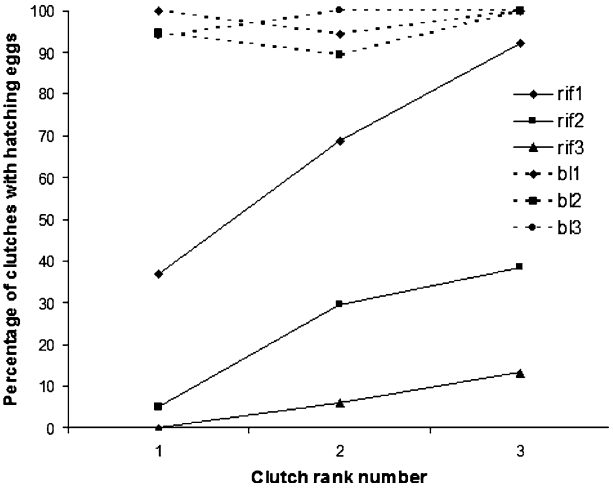

Fig. 2 Hatching percentage of three consecutive clutches of *Folsomia candida* in the rifampicin (rif1, rif2, rif3) and control (bl1, bl2, bl3) treatment

Table 2 Number (and percentage) of females with viable eggs in control and rifampicin-treated *F. candida*

	Number of infected females	Number of females with viable eggs (%)	Number of non-infected females	Number of females with viable eggs (%)
Control1	16	16 (100)	0	–
Control2	18	18 (100)	0	–
Control3	15	15 (100)	0	–
Control	**49**	**49 (100)**	**0**	**–**
Rifampicin1	13	12 (92.3)	0	–
Rifampicin2	6	5 (83.3)	7	0 (0)
Rifampicin3	5	2 (40)	10	0 (0)
Rifampicin	**24**	**19 (79.2)**	**17**	**0 (0)**
Heat	**11**	**8 (72.7)**	**6**	**0 (0)**

All control females are *Wolbachia*-infected, whereas among the rifampicin-treated females infected and non-infected females are observed. None of the non-infected females produced viable eggs. Totals are given in *bold*

verified using PCR (again with 16S rDNA primers). The efficiency of heat treatment in removing *Wolbachia* was 35% (6 out of 17), which is less efficient than rifampicin treatment. The effect of the heat treatment, however, was highly comparable to that of rifampicin: all six uninfected individuals failed to produce any viable offspring (Table 2). Of the 11 heat-treated, but infected individuals, 8 produced viable offspring. The distribution of successful egg hatching over infected and non-infected individuals deviated significantly from random (Fisher's Exact test, $P = 0.009$). We may therefore conclude that the decrease in hatching success is the result of removing *Wolbachia* rather than a toxic effect of rifampicin.

Quantitative PCR analysis of *ftsZ* DNA of all heat and antibiotic treated animals revealed that *Wolbachia* densities differed among the three predefined groups (Fig. 3) (Kruskal-Wallis test, $P < 0.001$). Uninfected females without hatching eggs had the lowest

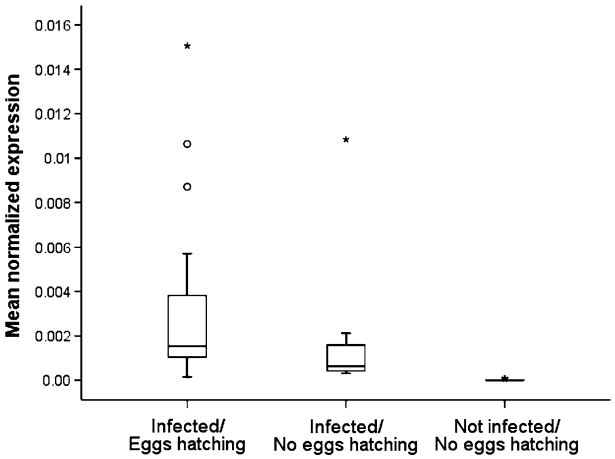

Fig. 3 Relative abundance of *Wolbachia ftsZ* DNA (estimated as Mean normalized expression) of three groups of animals: infected individuals with hatching eggs (n = 24); Infected individuals without hatching eggs (n = 7); Uninfected individuals without hatching eggs (n = 23). Each box shows the median, quartiles, and extreme values within a category. The symbols ° and * denote outliers and extreme cases, respectively; they were included in the analyses. Categories of infected and non-infected individuals were based on 16S rDNA PCR screening. Four individuals could not be included in this analysis due to failure of quantitative PCR amplification reactions

bacterial density followed by infected females without hatching eggs and infected females with hatching eggs. Although on average infected animals without hatching eggs showed low relative infection rates (Fig. 3), these infection rates were not significantly different from the group with hatching eggs (Mann-Whitney test, $P = 0.09$).

Discussion

Here we demonstrated that *Wolbachia* is essential for successful reproduction in the soil arthropod *F. candida*. Treatment with antibiotics or heat treatment resulted in the loss of *Wolbachia*-infection without adverse effects on oviposition. However, the eggs produced by these uninfected females were unviable. Although *Wolbachia* is often described as a facultative endosymbiont (Dale and Moran 2006), our data show that *Wolbachia*-infection is obligatory for offspring survival of the springtail *F. candida*.

To our knowledge this is one of the few cases of obligatory *Wolbachia* infection in arthropods. Removal of *Wolbachia* is known to result in functional sterility in haplo-diploid species (Pannebakker et al. 2005; Zchori-Fein et al. 1992), perhaps as the result of mutation-accumulation over time (Carson et al. 1982; Pijls et al. 1996). A direct interaction between *Wolbachia* and its host was demonstrated in the hymenopteran parasitoid *Asobara tabida*, where *Wolbachia* is necessary for oogenesis (Dedeine et al. 2005; Dedeine et al. 2001). The complete dependence of *A. tabida* on *Wolbachia* is probably due to bacterial manipulation of apoptotic pathways during oogenesis (Pannebakker et al. 2007). Loss of *Wolbachia* in this species results in apoptosis of nurse cells before the oocytes are mature. Our data show a similar direct dependence on *Wolbachia* in *F. candida* but suggest a different mechanism since *F. candida* produces clutches of eggs that appear healthy (i.e., all freshly laid eggs were of normal size and whitish, and turned brownish after a couple of

hours, as commonly observed for *F. candida*). To test the generality of the mechanisms underlying different host-*Wolbachia* interactions, more instances of obligatory *Wolbachia*-infection need to be studied.

In his influential review on the biology of *Wolbachia*, Werren (1997) already proclaimed that the discovery of parthenogenesis-inducing bacteria outside the Hymenoptera would be an important finding. While since then some studies have reported *Wolbachia*-induced parthenogenesis in Thysanoptera (Arakaki et al. 2001) and Coleoptera (Zchori-Fein et al. 2006), all reports concern haplo-diploid species. *F. candida* belongs to a family with a diploid–diploid sex determination system (Dallai et al. 1999), yet, we can not be conclusive on the role of *Wolbachia* in *F. candida*'s parthenogenetic reproduction. Nevertheless our results add to the appealing hypothesis proposed by Riparbelli et al. (2006). These authors suggested that *Wolbachia*-induced modifications of the zygotic spindle formation and chromosome segregation during meiosis could result in the restoration of diploidy of oocytes, which may induce parthenogenesis (Riparbelli et al. 2006). This would imply that in the absence of *Wolbachia* (e.g., due to antibiotic treatment as presented here) oocytes remain haploid, and therefore unviable. Additional experiments are needed to validate this hypothesis.

In another *Wolbachia*-infected, haplo-diploid species, *Coccotrypes dactyliperda*, females also failed to produce eggs after antibiotic treatment (Zchori-Fein et al. 2006). However, in this case the causative agent is yet to be determined since *C. dactyliperda* is also infected with *Rickettsia*. The male-biased sex-ratios of this host species and the inhibition of oogenesis associated with antibiotic treatment may well be caused by *Rickettsia*, since *Rickettsia* can act as a male-killing agent (Lawson et al. 2001) and have recently been found to be obligatory endosymbionts (Perotti et al. 2006). *Rickettsia* are essential for early development of the oocyte in the parthenogenetic booklice *Liposcelis bostrychophila* (Perotti et al. 2006). Other endosymbiotic bacteria than *Wolbachia* are reported from *F. candida* as well: based on bacterial screenings using staining and PCR (Czarnetzki and Tebbe 2004a, b) most likely *Rickettsiella*-like bacteria (RLO) were detected in the gut and fat-body of *F. candida* (GenBank: AF327558). The *F. candida* strain used in our study may be infected by RLO as well. Bioinformatic analysis of a recently published EST dataset of the same strain (Timmermans et al. 2007) revealed two sequences (GenBank: EV480534 and GE647879) which each harbored a fragment that showed high similarity to the published RLO sequence. Still, in our experiments with *F. candida* we can be reasonably sure that the effect of rifampicin and heat treatment can be attributed to *Wolbachia,* because these RLO seem to be restricted to the gut and the fat-body of *F. candida* (they are not associated with gonads (Czarnetzki and Tebbe 2004a)). Furthermore, previous work suggested that these RLO are not obligatory for *F. candida* (Czarnetzki and Tebbe 2004b).

Obligate symbionts such as *Buchnera* and *Baumannia* often provide their hosts with some essential nutrients (Zientz et al. 2004). More than a tenth of the strongly reduced genome of obligate symbionts is dedicated to host nutrient provision such as biosynthetic pathways for amino acids or essential cofactors (Dale and Moran 2006; Wu et al. 2006). Obligate pathogens such as *Rickettsia* and *Mycoplasma* do not retain such genes and there is no evidence that they provide any advantage to their hosts (Dale and Moran 2006). Since uninfected *F. candida* females survive well, and produce as many eggs as infected individuals, there is no evidence that they are dependent on nutritious substances from *Wolbachia*. Instead, we have shown that the collembolan *F. candida* is held hostage by its *Wolbachia* endosymbiont, through its effects acting specifically on egg development or meiosis.

One of our more salient results is the increase in hatching success in consecutive egg clutches of antibiotic-treated individuals over time. An earlier report also showed variation in *F. candida* egg hatchability under antibiotic treatment (Vandekerckhove et al. 2000), but this study did not control for potential toxic effects of antibiotic treatments, causing a decline in egg hatchability. In our study, we eliminated confounding effects of antibiotic treatment by stopping rifampicin treatment before reproduction was measured. Furthermore, since the animals were kept individually, no new infection by horizontal transfer could have taken place, leaving only the possibility of restoration of bacterial densities over time (see also Casiraghi et al. 2002). These observations provide circumstantial evidence for the hypothesis that reproduction in this Collembola species requires a critical threshold of bacterial density. The bacterial dosage model has been confirmed for cytoplasmic incompatibility (Bordenstein et al. 2006; Bourtzis et al. 1996; Breeuwer and Werren 1993; Noda et al. 2001; Poinsot et al. 1998; Veneti et al. 2003) and male killing *Wolbachia* (Hurst et al. 2000). Although we were not able to show that infected females without hatching eggs had significantly lower bacterial densities than infected females with hatching eggs, we discuss two possible reasons why this does not invalidate the bacterial dosage model for *F. candida*. First, bacterial densities are probably not homogeneous throughout the body of its host, so that high quantitative PCR values may be caused by clusters of bacteria in body parts other than the ovaries, where bacterial density is critical for egg hatching. Second, even in animals classified as non-infected based on regular PCR, quantitative PCR still showed the presence of low numbers of *Wolbachia* bacteria. Density of *Wolbachia* infection therefore seems to be a continuous variable rather than a simple binomial state, consistent with the assumptions of the bacterial dosage model.

We do not know what regulates bacterial densities, nor the nature of the chemical factors produced by *Wolbachia* necessary to enable egg hatching. However, the recent establishment of an EST database for *F. candida* (Timmermans et al. 2007) will greatly facilitate a more detailed molecular analysis of the genetic changes produced by *Wolbachia* infection.

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References

Arakaki N, Miyoshi T, Noda H (2001) *Wolbachia*-mediated parthenogenesis in the predatory thrips *Fanklintothrips vespiformis* (Thysanoptera: Insecta). Proc R Soc Lond B Biol Sci 268:1011–1016. doi:10.1098/rspb.2001.1628

Bordenstein SR, Marshall ML, Fry AJ et al (2006) The tripartite associations between bacteriophage, *Wolbachia*, and arthropods. PLoS Pathog 2:384–393

Bouchon D, Rigaud T, Juchault P (1998) Evidence for widespread *Wolbachia* infection in isopod crustaceans: molecular identification and host feminization. Proc R Soc Lond B Biol Sci 265:1081–1090. doi:10.1098/rspb.1998.0402

Bourtzis K, Nirgianaki A, Markakis G et al (1996) *Wolbachia* infection and cytoplasmic incompatibility in *Drosophila* species. Genetics 144:1063–1073

Breeuwer JAJ, Werren JH (1993) Cytoplasmic incompatibility and bacterial density in *Nasonia vitripennis*. Genetics 135:565–574

Carson HL, Chang LS, Lyttle TW (1982) Decay of female sexual behavior under parthenogenesis. Science 218:68–70. doi:10.1126/science.218.4567.68

Casiraghi M, McCall JW, Simoncini L et al (2002) Tetracycline treatment and sex-ratio distortion: a role for *Wolbachia* in the moulting of filarial nematodes? Int J Parasitol 32:1457–1468. doi:10.1016/S0020-7519(02)00158-3

Czarnetzki AB, Tebbe CC (2004a) Detection and phylogenetic analysis of Wolbachia in Collembola. Environ Microbiol 6:35–44. doi:10.1046/j.1462-2920.2003.00537.x

Czarnetzki AB, Tebbe CC (2004b) Diversity of bacteria associated with Collembola—a cultivation-independent survey based on PCR-amplified 16S rRNA genes. FEMS Microbiol Ecol 49:217–227. doi:10.1016/j.femsec.2004.03.007

Dale C, Moran NA (2006) Molecular interactions between bacterial symbionts and their hosts. Cell 126:453–465. doi:10.1016/j.cell.2006.07.014

Dallai R, Fanciulli PP, Frati F (1999) Chromosome elimination and sex determination in springtails (Insecta, Collembola). J Exp Zool 285:215–225. doi:10.1002/(SICI)1097-010X(19991015)285:3<;215::AID-JEZ4>;3.0.CO;2-5

Dedeine F, Vavre F, Fleury F et al (2001) Removing symbiotic *Wolbachia* bacteria specifically inhibits oogenesis in a parasitic wasp. Proc Natl Acad Sci USA 98:6247–6252. doi:10.1073/pnas.101304298

Dedeine F, Bouletreau M, Vavre F (2005) *Wolbachia* requirement for oogenesis: occurrence within the genus *Asobara* (Hymenoptera, Braconidae) and evidence for intraspecific variation in *A. tabida*. Heredity 95:394–400. doi:10.1038/sj.hdy.6800739

Frati F, Negri I, Fanciulli PP et al (2004) High levels of genetic differentiation between *Wolbachia*-infected and non-infected populations of *Folsomia candida* (Collembola, Isotomidae). Pedobiol 48:461–468. doi:10.1016/j.pedobi.2004.04.004

Hurst GDD, Jiggins FM, von der Schulenburg JHG et al (1999) Male-killing *Wolbachia* in two species of insect. Proc R Soc Lond B Biol Sci 266:735–740. doi:10.1098/rspb.1999.0698

Hurst GDD, Johnson AP, von der Schulenburg JHG et al (2000) Male-killing *Wolbachia* in *Drosophila*: a temperature-sensitive trait with a threshold bacterial density. Genetics 156:699–709

Lawson ET, Mousseau TA, Klaper R et al (2001) *Rickettsia* associated with male-killing in a buprestid beetle. Heredity 86:497–505. doi:10.1046/j.1365-2540.2001.00848.x

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25:402–408. doi:10.1006/meth.2001.1262

Lo N, Casiraghi M, Salati E et al (2002) How many *Wolbachia* supergroups exist? Mol Biol Evol 19:341–346

Mallatt J, Giribet G (2006) Further use of nearly complete, 28S and 18S rRNA genes to classify Ecdysozoa: 37 more arthropods and a kinorhynch. Mol Phyl Evol 40:772–794. doi:10.1016/j.ympev.2006.04.021

Negri I, Pellecchia M, Mazzoglio PJ et al (2006) Feminizing *Wolbachia* in *Zyginidia pullula* (Insecta, Hemiptera), a leafhopper with an XX/XO sex-determination system. Proc R Soc Lond B Biol Sci 273:2409–2416. doi:10.1098/rspb.2006.3592

Noda H, Koizumi Y, Zhang Q et al (2001) Infection density of *Wolbachia* and incompatibility level in two planthopper species, *Laodelphax striatellus* and *Sogatella furcifera*. Insect Biochem Mol Biol 31:727–737. doi:10.1016/S0965-1748(00)00180-6

Normark BB (2003) The evolution of alternative genetic systems in insects. Annu Rev Entomol 48:397–423. doi:10.1146/annurev.ento.48.091801.112703

Pannebakker BA, Schidlo NS, Boskamp GJF et al (2005) Sexual functionality of *Leptopilina clavipes* (Hymenoptera : Figitidae) after reversing *Wolbachia*-induced parthenogenesis. J Evol Biol 18:1019–1028. doi:10.1111/j.1420-9101.2005.00898.x

Pannebakker BA, Loppin B, Elemans CPH et al (2007) Parasitic inhibition of cell death facilitates symbiosis. Proc Natl Acad Sci USA 104:213–215. doi:10.1073/pnas.0607845104

Perotti MA, Clarke HK, Turner BD et al (2006) *Rickettsia* as obligate and mycetomic bacteria. FASEB J 20:2372. doi:10.1096/fj.06-5870fje

Pijls J, van Steenbergen HJ, van Alphen JJM (1996) Asexuality cured: The relations and differences between sexual and asexual Apoanagyrus diversicornis. Heredity 76:506–513. doi:10.1038/hdy.1996.73

Poinsot D, Bourtzis K, Markakis G et al (1998) *Wolbachia* transfer from *Drosophila melanogaster* into *D. simulans*: host effect and cytoplasmic incompatibility relationships. Genetics 150:227–237

Quinn GP, Keough MJ (2002) Experimental design and data analysis for biologists. Cambridge University Press, Cambridge

Riparbelli MG, Giordano R, Callaini G (2006) Centrosome inheritance in the parthenogenetic egg of the collembolan *Folsomia candida*. Cell Tissue Res 326:861–872. doi:10.1007/s00441-006-0253-x

Simon P (2003) Q-Gene: processing quantitative real-time RT-PCR data. Bioinformatics 19:1439–1440. doi:10.1093/bioinformatics/btg157

Stouthamer R, Luck RF, Hamilton WD (1990) Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera, Trochogrammatidae) to revert to sex. Proc Natl Acad Sci USA 87:2424–2427. doi:10.1073/pnas.87.7.2424

Stouthamer R, Breeuwer JAJ, Hurst GDD (1999) *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu Rev Microbiol 53:71–102. doi:10.1146/annurev.micro.53.1.71

Timmermans MJTN, Marien J, Roelofs D, van Straalen NM, Ellers J (2004) Evidence for multiple origins of *Wolbachia* infection in springtails. Pedobiologia (Jena) 48:469–475. doi:10.1016/j.pedobi.2004.07.008

Timmermans MJTN, de Boer ME, Nota B, de Boer TE, Marien J, Klein-Lankhorst RM, van Straalen NM, Roelofs D (2007) Collembase: a repository for springtail genomics and soil quality assessment. BMC Genomics 8:341

Timmermans MJTN, Roelofs D, Marien J, van Straalen NM (2008) Revealing pancrustacean relationships: phylogenetic analysis of ribosomal protein genes places Collembola (springtails) in a monophyletic Hexapoda and reinforces the discrepancy between mitochondrial and nuclear DNA markers. BMC Evol Biol 8:83

Vandekerckhove TTM, Watteyne S, Willems A et al (1999) Phylogenetic analysis of the 16S rDNA of the cytoplasmic bacterium *Wolbachia* from the novel host *Folsomia candida* (Hexapoda, Collembola) and its implications for wolbachial taxonomy. FEMS Microbiol Lett 180:279–286

Vandekerckhove TTM, Watteyne S, Bocksoen L et al (2000) *Wolbachia*-induced thelytoky in the springtail *Folsomia candida* (Hexapoda, Collembola).In: Proceedings of the first international Wolbachia conference, kolymbari 35–36

Veneti Z, Clark ME, Zabalou S et al (2003) Cytoplasmic incompatibility and sperm cyst infection in different *Drosophila-Wolbachia* associations. Genetics 164:545–552

Weeks AR, Breeuwer JAJ (2001) Wolbachia-induced parthenogenesis in a genus of phytophagous mites. Proc R Soc Lond B Biol Sci 268:2245–2251. doi:10.1098/rspb.2001.1797

Werren JH (1997) Biology of Wolbachia. Annu Rev Entomol 42:587–609. doi:10.1146/annurev.ento.42.1.587

Werren JH, Hurst GDD, Zhang W et al (1994) Rickettsial relative associated with male killing in the ladybird beetle (*Adalia bipunctata*). J Bacteriol 176:388–394

Werren JH, Zhang W, Guo LR (1995) Evolution and phylogeny of Wolbachia—reproductive parasites of arthropods. Proc R Soc Lond B Biol Sci 261:55–63. doi:10.1098/rspb.1995.0117

Wu D, Daugherty SC, Van Aken SE et al (2006) Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. PLoS Biol 4:1079–1092. doi:10.1371/journal.pbio.0040188

Zchori-Fein E, Roush RT, Hunter MS (1992) Male production induced by antibiotic-treatment in *Encarsia formosa* (Hymenoptera, Aphelinidae), an asexual Species. Experientia 48:102–105. doi:10.1007/BF01923619

Zchori-Fein E, Borad C, Harari AR (2006) Oogenesis in the date stone beetle, *Coccotrypes dactyliperda*, depends on symbiotic bacteria. Physiol Entomol 31:164–169. doi:10.1111/j.1365-3032.2006.00504.x

Zientz E, Dandekar T, Gross R (2004) Metabolic interdependence of obligate intracellular bacteria and their insect hosts. Microbiol Mol Biol Rev 68:745. doi:10.1128/MMBR.68.4.745-770.2004